Baicalein induces apoptosis and autophagy of breast cancer cells via inhibiting PI3K/AKT pathway in vivo and vitro

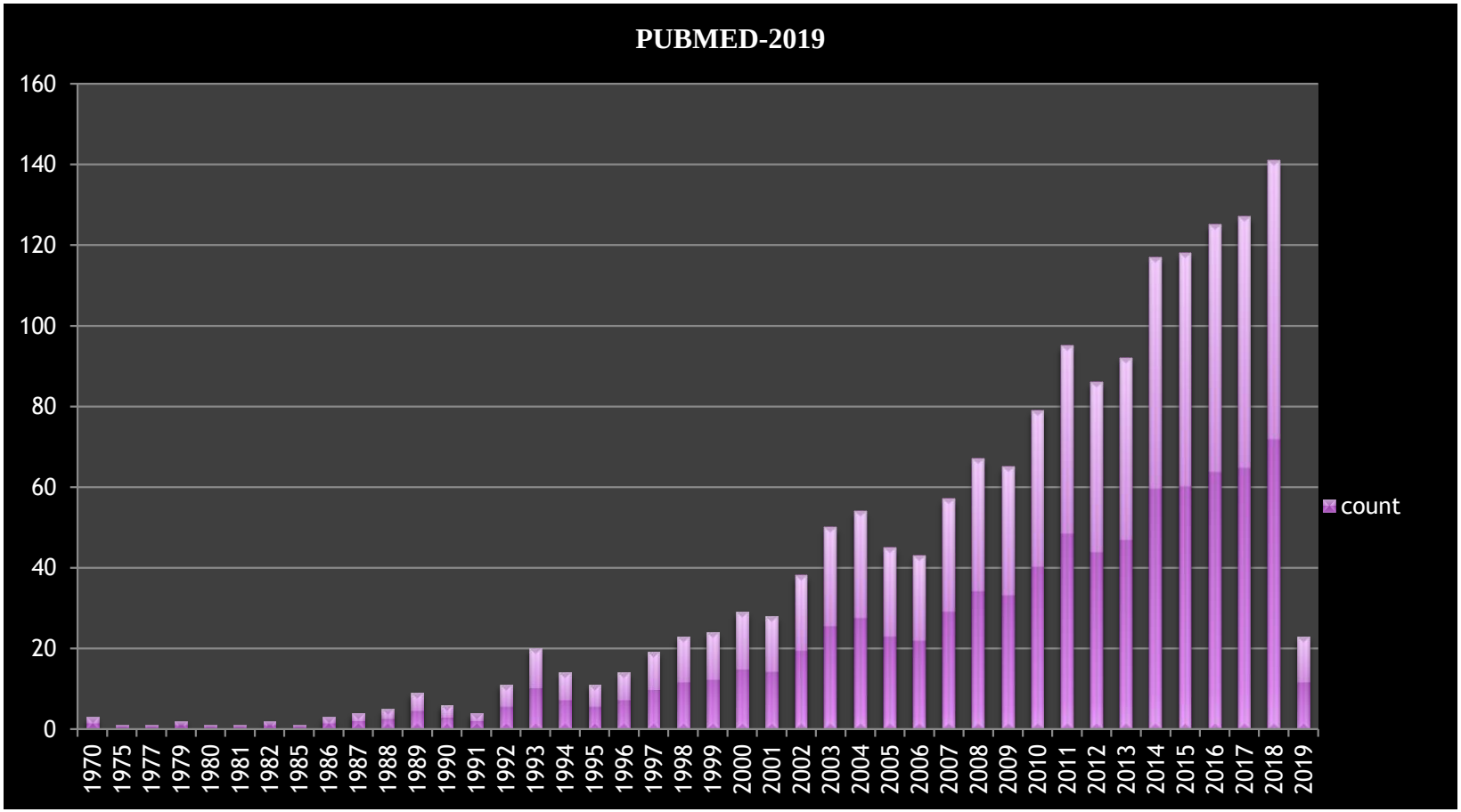
Presented by: Leila Soufi

M.Sc Student of Medical Biotechnology
Paramedical Sciences Faculty

Qazvin University of Medical Sciences

Supervisor: Dr.Gheibi

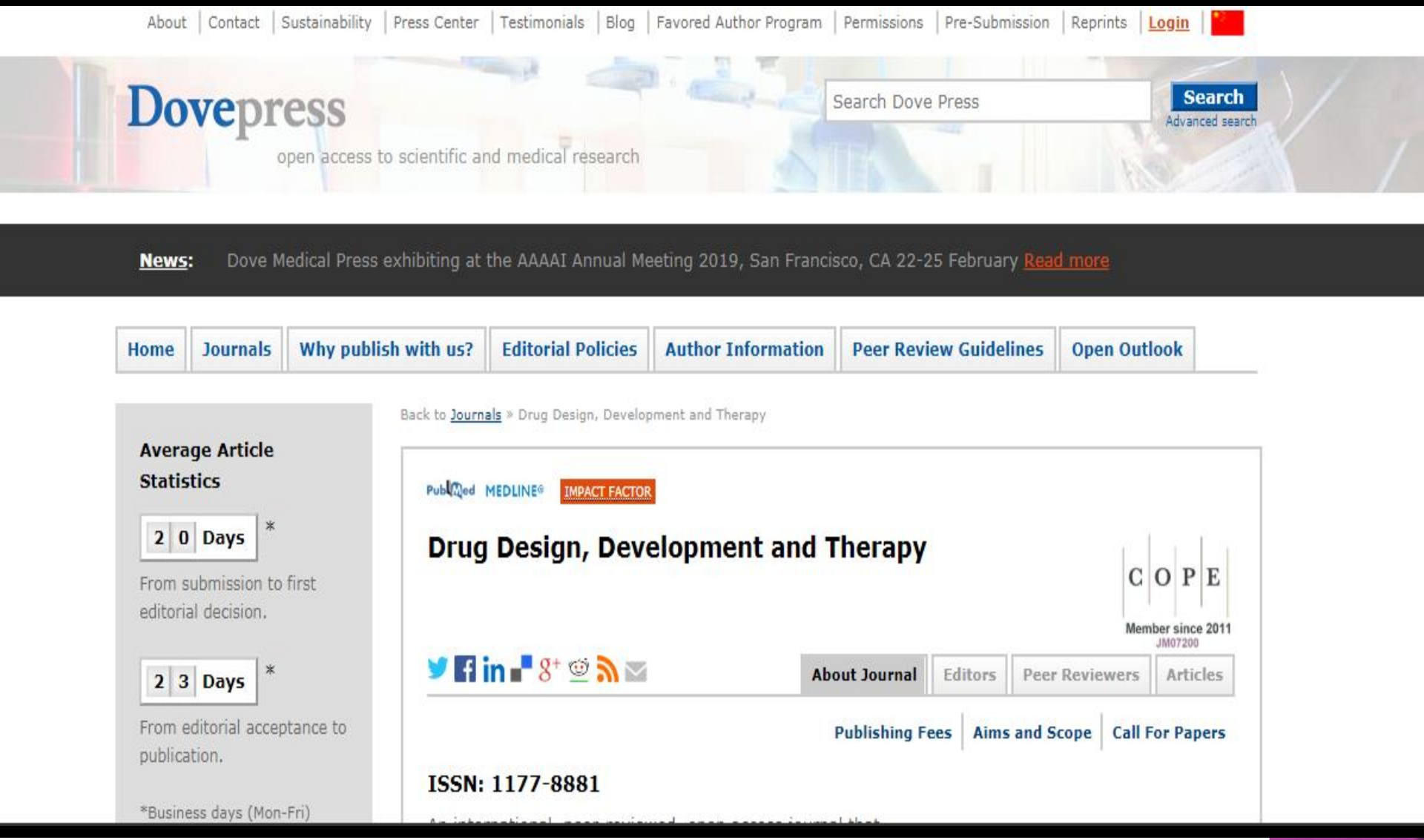
2019


PUBMED-2019
160
140
120
100
80
60
40
20
0
1970
1975
1977
1979
1980
1981
1982
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
count

“List of Contents”

- Journal information
- Introduction
- Materials & methods
- Results
- Discussion
- Conclusion

Journal information



Journal information

2 3 Days *

From editorial acceptance to publication.

*Business days (Mon-Fri)

Rejection Rate

5 7 % *

The above percentage of manuscripts have been rejected in the last 12 months.

Submit Manuscript

Journal Email Alerts

Signup for Alerts

About Dove Press

Open access peer reviewed

About Journal | Editors | Peer Reviewers | Articles

Publishing Fees | Aims and Scope | Call For Papers

ISSN: 1177-8881

An international, peer-reviewed, open access journal that spans the spectrum of drug design and development through to clinical applications. The journal is characterized by the rapid reporting of application notes, reviews, original research and clinical studies in all therapeutic areas. Clinical outcomes, patient safety, and programs for the development and effective, safe, and sustained use of medicines will be a feature of the journal.

This journal is a member of and subscribes to the principles of the Committee on Publication Ethics (COPE).

Journal Impact Factor: 2.935 (5 year impact 3.047)

Editor-in-Chief: Prof. Dr. Frank Boeckler

Indexed online in:

- PubMed and PubMed Central (*Drug Des Devel Ther*)
- MedLine on MEDLARs
- Science Citation Index Expanded (also known as SciSearch®)
- Journal Citation Reports/Science Edition

Drug Design, Development and Therapy

Q1 Drug Discovery

best quartile

Journal information

Journal Email Alerts

Signup for Alerts

About Dove Press

Open access peer-reviewed scientific and medical journals.

Learn more

Open Access

Dove Medical Press is a member of the OAI.

Learn more

Reprints

Bulk reprints for the pharmaceutical industry.

Indexed online in:

- PubMed and PubMed Central (*Drug Des Devel Ther*)
- MedLine on MEDLARs
- Science Citation Index Expanded (also known as SciSearch®)
- Journal Citation Reports/Science Edition
- Biological Abstracts
- BIOSIS Previews
- Embase, from 2009 (Correct as at December 8, 2016)
- Scopus, from 2009 (Correct as at December 8, 2016)
- Directory of Open Access Journals (DOAJ)
- OAIster: The Open Access Initiative

Related journals you may also be interested in:

Adolescent Health, Medicine and Therapeutics

Biologics: Targets and Therapy

Clinical Interventions in Aging

Clinical Pharmacology: Advances and Applications

ClinicoEconomics and Outcomes Research

Core Evidence

Introduction

- **Breast cancer:**

- **One of the most commonly occurring female malignant tumors.**
- **Increased incidence and much younger onset age recently.**

- **Although there have been significant advances in screening ,surgery, and chemo radiotherapy techniques, the prognosis of patients remains little known.**

- **Hence, it is urgent to provide a new therapeutic strategy in cancer therapy.**

Introduction

- **Baicalein:**

- **A bioactive component extracted from the root of Scutellaria baicalensis Georgi.**
- **Anti-tumor, anti-inflammatory, anti-cardiovascular disease, & antimicrobial activities**

- **Numerous studies: anti-tumor properties of baicalein in many types of human cancer cell lines both in vitro & in vivo.**

- **Inhibiting cell proliferation.**
- **Inducing cell apoptosis (activating the caspase cascade & mitochondrial apoptotic pathway, DNA fragmentation in malignant cells).**

Introduction

- **Past studies: Baicalein-induced apoptosis & autophagy via PI3K/AKT signaling pathway inhibition in renal carcinoma, glioma, epidermoid carcinoma, and bladder cancer.**

- **Few studies: were on latent molecular mechanism of anticancer activity of baicalein on human breast cancer cells.**

- **Present study purpose: ascertain potential mechanisms through which baicalein induces apoptosis & autophagy in MCF7 and MDA-MB-231 cells: (PI3K/AKT inhibition).**

- **PI3K/AKT signaling pathway play roles in :**
- **Mammalian cell proliferation, differentiation, apoptosis, autophagy , & survival.**
- **Survival of human cancer cells in vitro.**
- **Carcinogenicity, invasion and metastasis of human cancer cells in vivo.**

Materials & methods

- **Cell culture**
- **MCF-7 and MDA-MB-231 cells: were cultured in DMEM supplemented with 10% FBS, 50 IU/mL penicillin, and 50 µg/mL streptomycin and maintained in humidified 5% CO2 air at 37°C.**

- **Animals:**
- **Female BALB/c nude mice (3–6 weeks old, body weight 18–20 g) (from Experimental Animal Center of Xi'an Jiaotong University).**

Materials & methods

Morphological cell changes

- Cells were seeded in 12-well plates at a density of 3×10^5 cells/well and grown for 24 hours.
- Cells were treated with various concentrations (0, 10, 20, and 40 μM). of baicalein.
- grown at 37°C, in 5% CO2 and 95% air for 24 hours.
- For examining morphological changes, cells were observed and photographed under a phase-contrast microscope.

Materials & methods

MTT assay

- The cell viability was assessed by MTT assay.
- Cells were seeded into 96-well plates (4×103 cells/well) .
- Incubated in 5% CO2 air at 37°C.
- After 12h incubation, the cells were treated with different concentrations of baicalein (0, 10, 20, and 40 μM) for 24, 48, and 72 hours.
- MTT (20 μL of 5 mg/mL) was added to each well and incubated at 37°C for 4 hours.
- The formazan crystals were dissolved in DMSO, then incubated for 10 min.
- The OD was recorded at 490 nm on a microplate reader.
- The inhibition ratio (IR) = (1-mean OD value of experimental group/mean OD value of control group)×100%.

Materials & methods

Colony formation assay

- Cells (1000 cells/well) were seeded into 6-well plates.
- Treated with baicalein (0, 10, 20, and 40 μM) for 48 hours.

- Then cultured in DMEM at 37°C for 14 days.
- During this period, the cells were washed with PBS every 3 days.

- Fixed with paraformaldehyde (4%, 15 minutes)
- Stained with crystal violet (0.1%, 15 minutes) and the numbers of colonies with >50 cells were counted with an Olympus digital camera.

Materials & methods

Hoechst 33258 staining for apoptosis

- Cells (2×105 cells/well) were seeded in 12-well plates for 12 hours
- Treated with various concentrations of baicalein (0, 10, 20, and 40 μM) for 48 hours

- Washed with PBS, and then fixed in paraformaldehyde (4%) for 15 minutes at room temperature.
- Cells were stained with 100 μL Hoechst 33258 in PBS for 15 minutes at room temperature.

- The stained cells were visualized from randomly selected fields under a fluorescence microscope (The nuclear condensation and fragmentation of cells were identified as the apoptotic cells).

Materials & methods

Measurement of mitochondrial membrane potential (ΔΨm)

- Cells were seeded in 12-well plates at a density of 2×10^5 cells/well for 12 hours.
- Treated with different concentrations of baicalein (0, 10, 20, and 40 μM) for 48h.

- Cells were harvested and washed with PBS and resuspended in JC-1 at 37°C for 30 minutes in the dark.
- The stained cells were analyzed by a fluorescence microscope.

Materials & methods

AO staining

- MCF-7 and MDA-MB-231 cells (1×105 cells/well) were suspended and seeded in a 12-well plate and incubated overnight.

- Treated with baicalein (0, 10, 20, and 40 μM) for 48 hours.
- Stained with AO (1 μg/mL) for 15 minutes in the dark

- Washed with PBS, and visualized under a fluorescence microscope.

Materials & methods

Transmission electron microscopy (TEM) observation

(to observe the ultrastructure)

- **Cells were incubated with 40 μg/mL baicalein for 48 hours.**
- **Then cells were fixed in ice-cold glutaraldehyde in 0.1 mol/L phosphate buffer (PH 7.4) overnight.**
- **Fixed in 1% osmium tetroxide and dehydrated.**
- **Cells were impregnated with Epon.**
- **The ultrathin sections were contrasted with uranyl acetate and lead citrate for electron microscopy.**
- **Electron micrographs were observed through a transmission electron microscope.**

Materials & methods

Flowcytometry analysis

- **Treatment with various concentrations (0, 10, 20, and 40 µM) of baicalein for 48h.**
- **Cells were collected and washed twice in PBS at room temp.**
- **Cells (1×10^6) were resuspended in 100 µL annexin-V binding buffer solution.**
- **5 µL annexin V-FITC and 5 µL propidium iodide (PI) were added.**
- **Incubated at room temp. for 15 min in the dark.**
- **The percentage of apoptotic cells was analyzed by flowcytometry.**

Materials & methods

Quantitative real-time PCR(qRT-PCR)

- Cells were treated with various concentrations (0, 10, 20, and 40 μM) of baicalein for 48 hours.
- Total cell RNA was extracted by Trizol according to the manufacturer's instructions.
- RNA was then reverse transcribed into cDNA with RevertAid First-Strand cDNA Synthesis Kit.
- Then qRT-PCR reaction system was prepared following the manufacturer's instructions given in the SYBR®Premix Ex TaqII RT-PCR Kit (TaKaRa) using 100 ng cDNA.
- Each sample was analyzed by using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific).

Materials & methods

Table 1 Primers used for qRT-PCR analysis

Gene	Primer sequence	
	Forward (5′–3′)	Reverse (5′–3′)
Bcl-2	TCGCCCTGTGGATGACTGA	CAGAGACAGCCA GGAGAAATCA
Bax	GCGATGAACTGGACAACA ACAT	TAGCAAAGTAGAAAAGGGCAACC
LC3	GAGTGGAAGATGTCCGGCTC	CCAGGAGGAAGAAGGCTTGG
BECN1	CGGGATCCATGGAAGGGTCTAAGACGTCC	CGGAATTCTCATTTGTTATAAAATTGTGAGG
GAPDH	ATGCCAGTGAGCTTCCCGTCAGC	GGTATCGTGGAAGAACTCATGAC

Abbreviation: qRT-PCR, quantitative real-time PCR.

Materials & methods

Western blotting

- Cells cultured with various concentrations (0, 10, 20, and 40 μM) of baicalein for 24 hours, 48 hours, and 72 hours.
- Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes.
- Membranes were blocked with 5% non-fat milk for 2h at room temp. & incubated overnight at 4°C with a primary Ab (GAPDH, AKT, p-AKT, mTOR, p-mTOR, NF-κB, IκB, and p-IκB).
- Membranes were washed 3times with TBST & incubated with the appropriate HRP-conjugated secondary Ab for 2h at room temp.
- Protein bands were visualized using the chemiluminescence gel imaging system & quantitated using the Image-Pro Plus 6.0 software (GAPDH=internal control).

Materials & methods

Tumor xenograft study

- **Female BALB/c nude mice (3–6 weeks old).**
- **Xenografts were established by subcutaneous injection of MCF-7 ($1\times10_7$/200 µL) & MDA-MB-231 cells ($2\times10_6$ cells/200 µL) into the 2left breast pad of nude mice.**
- **When tumor size ≅100 mm3 ➔ mice were randomly divided into different groups (5 in each) and baicalein (100 mg/kg) or 1% carboxymethyl cellulose sodium was administered by intra-gastric gavage 1/day for 21 days.**
- **Tumor volume , growth and body weight were measured every 3days.**
- **Experiments were terminated at the 21st day and the animals were anesthetized and sacrificed. Tumors were removed for further analysis.**

Materials & methods

Immunohistochemistry

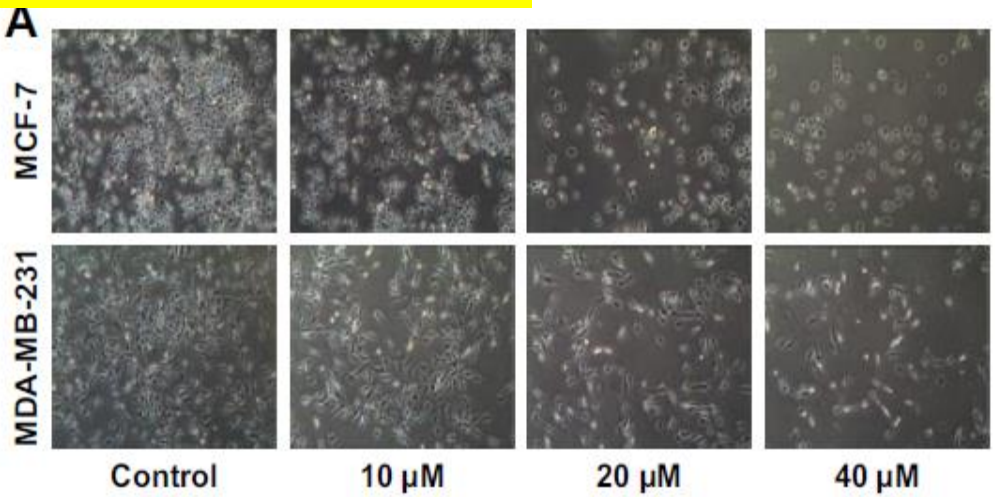
- Histological analysis was performed on tissue samples isolated from mouse xenografts.
- Briefly, 5 µm sections were cut from all paraffin blocks and stained for p-AKT, Bax, and LC3.
- Immunohistochemical staining was conducted using Histostain®-SP Kits according to the manufacturer's instructions.
- Sections were incubated with p-AKT, BAX, and LC3 primary antibody at 4°C overnight.
- Images of sections were visualized using a Zeiss microscope & analyzed by Image-Pro Plus 6.0.

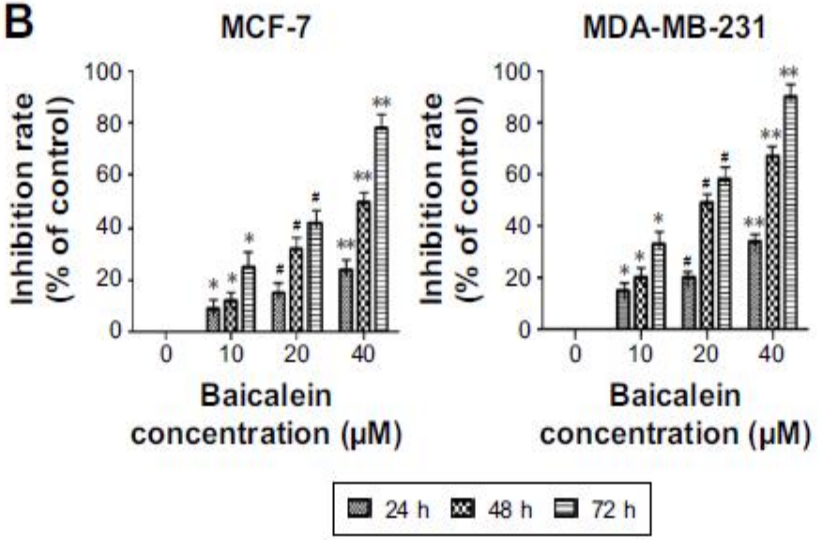
Results

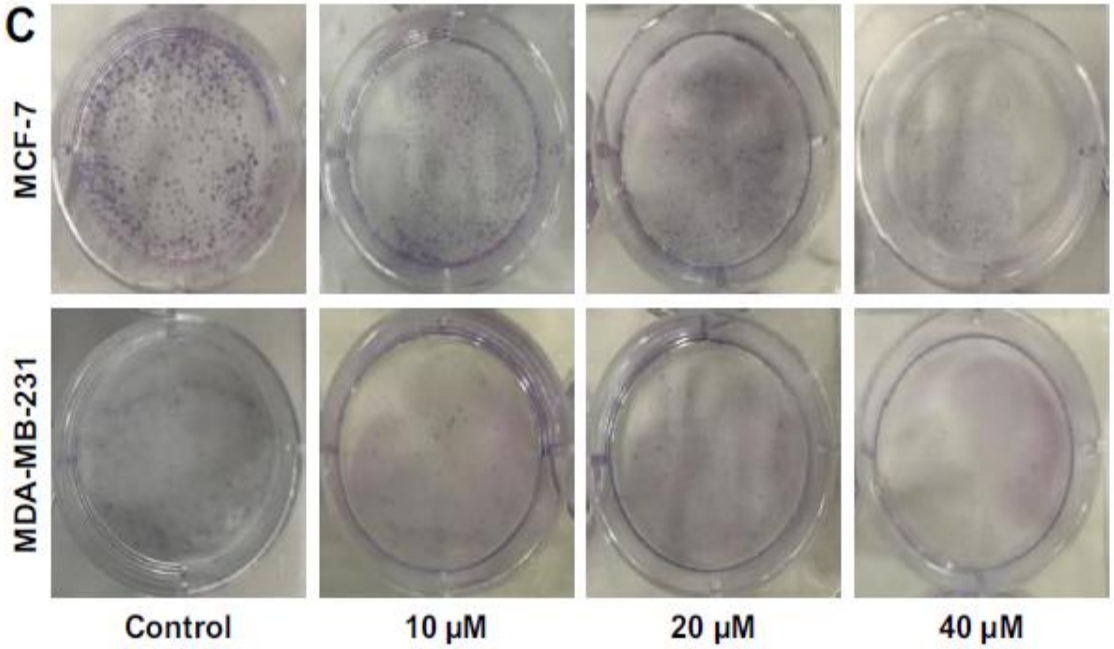
Baicalein inhibited the proliferation of breast cancer cells

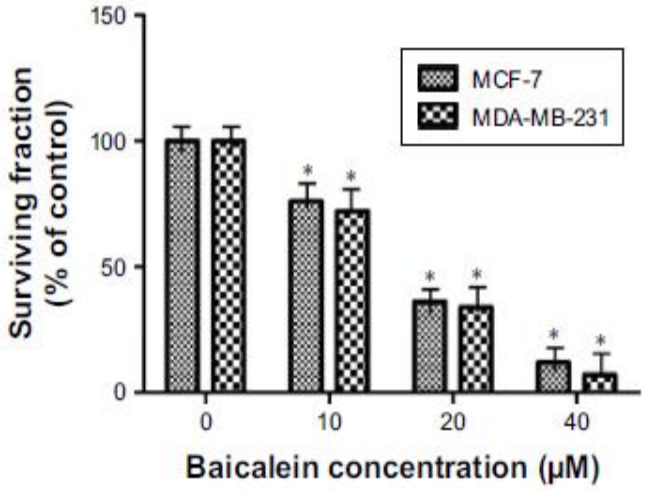
- **Phase-contrast Microscopy:**
- **MCF-7&MDA-MB-231 cells exposed to increasing concentrations of baicalein ➔ significant morphological changes, including cell shrinkage and blebbing (Fig.1A).**
- **MTT assay: baicalein significantly inhibited the proliferation of MCF-7 and MDA-MB-231 cells in a dose- and time-dependent manner (Fig.1B)**
- **Baicalein suppressed the colony formation of MCF-7 and MDA-MB-231 cells as shown by the plate colony formation assay (Fig.1C).**

Results


A
MCF-7
MDA-MB-231
Control
10 μM
20 μM
40 μM


B
MCF-7
MDA-MB-231
Inhibition rate (% of control)
Baicalein concentration (μM)
24 h
48 h
72 h


C
MCF-7
MDA-MB-231
Control
10 μM
20 μM
40 μM


Surviving fraction (% of control)
MCF-7
MDA-MB-231
Baicalein concentration (μM)

Results

Table 2 The IC50 values (μM) of baicalein in breast cancer cells

Cell lines	24 hours	48 hours	72 hours
MCF-7	51.06	20.12	13.98
MDA-MB-231	60.12	27.96	19.01

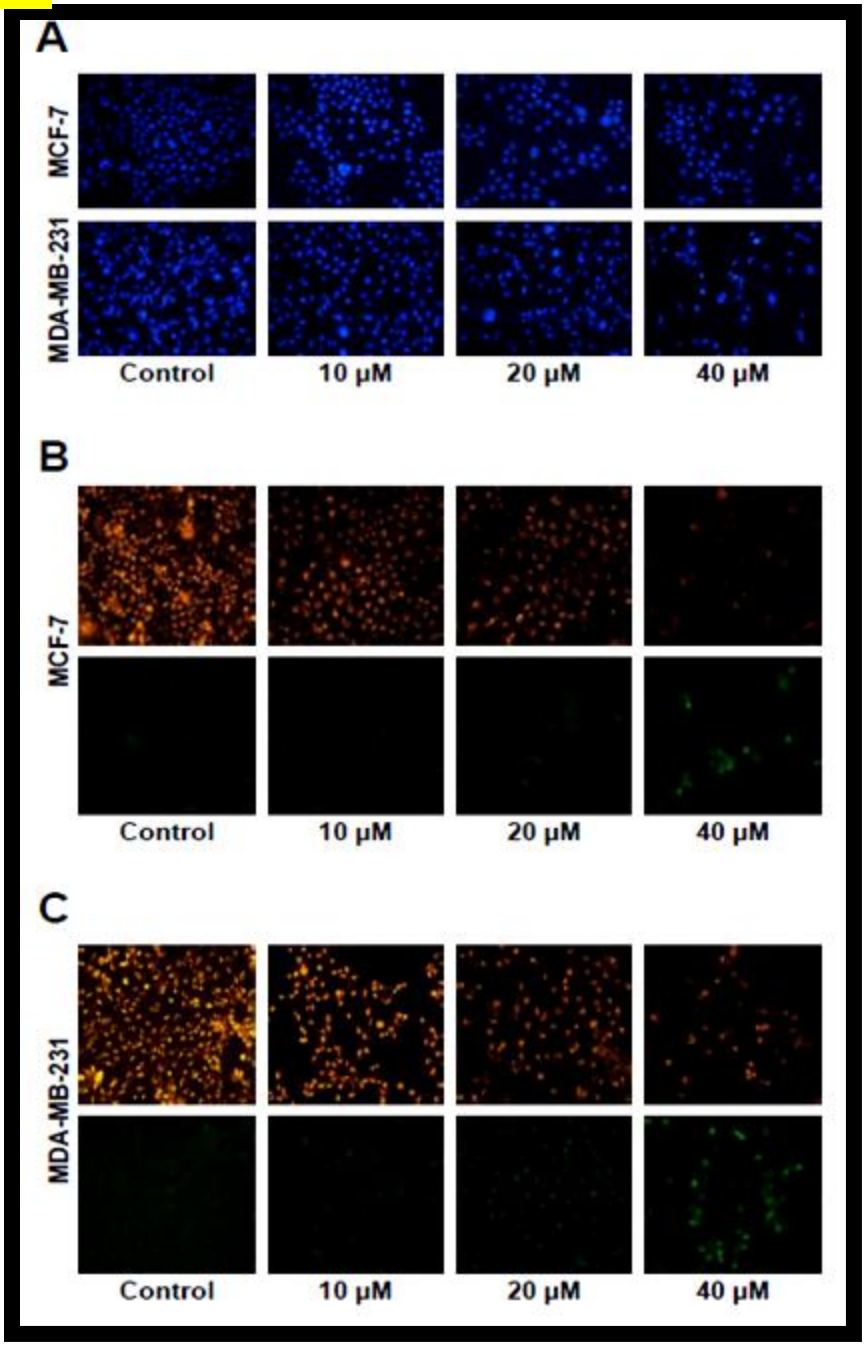
Abbreviation: IC50, 50% inhibition concentration.

Results

Baicalein induced cell apoptosis in breast cancer cells

- **Hoechst staining 33258 (sensitive to DNA & used to assess changes in cellular nuclear morphology), baicalein effects on nuclear condensation (Fig.2A).**
- **mitochondrial membrane potential (ΔΨm) changes (key events during drug-induced apoptosis were examined via fluorescent probe JC-1.**
- **normal cells: JC-1 accumulates & aggregates which characterized by red fluorescence in the mitochondria.**
- **During apoptosis: ΔΨm changes➔ JC-1 aggregates transform into monomers leading to loss of red fluorescence & ΔΨm changes are manifested via reduced ratio of red to green fluorescence.**

Results


A
MCF-7
MDA-MB-231
Control
10 μM
20 μM
40 μM
B
MCF-7
Control
10 μM
20 μM
40 μM
C
MDA-MB-231
Control
10 μM
20 μM
40 μM

Results

- **Annexin V-FITC/PI staining and flow cytometry:**
  - **Baicalein increases cell apoptosis in MCF-7 and MDA-MB-231 cells with an apoptotic rate estimated at:**

26.89%±0.96% and 27.73%±0.23% in 40 μM baicalein group,

20.55%±0.62% and 20.27%±0.36% in 20 μM baicalein group.

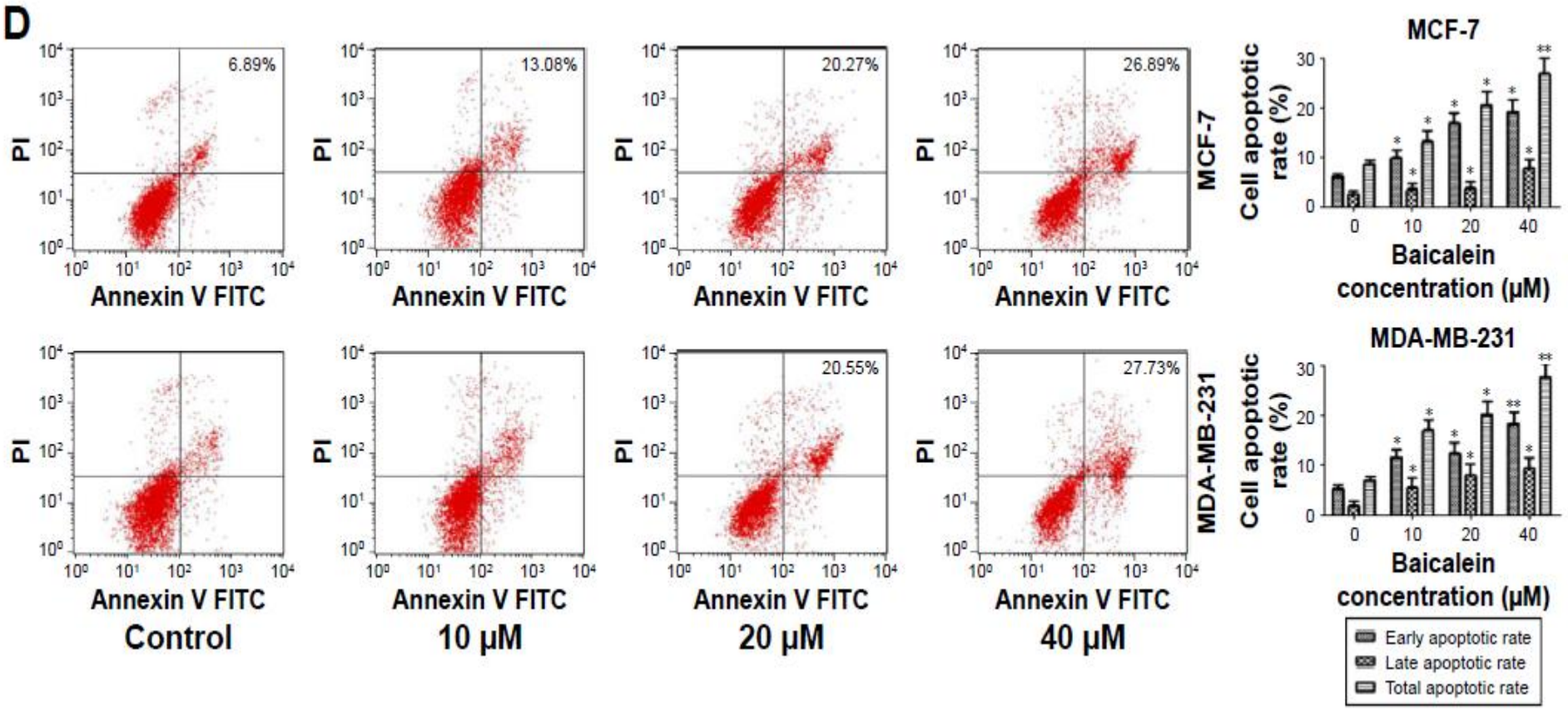
13.08%±0.78% and 16.94%±0.86% in 10 μM baicalein group.

and 8.62%±0.34% and 6.89%±0.65% in the control group.

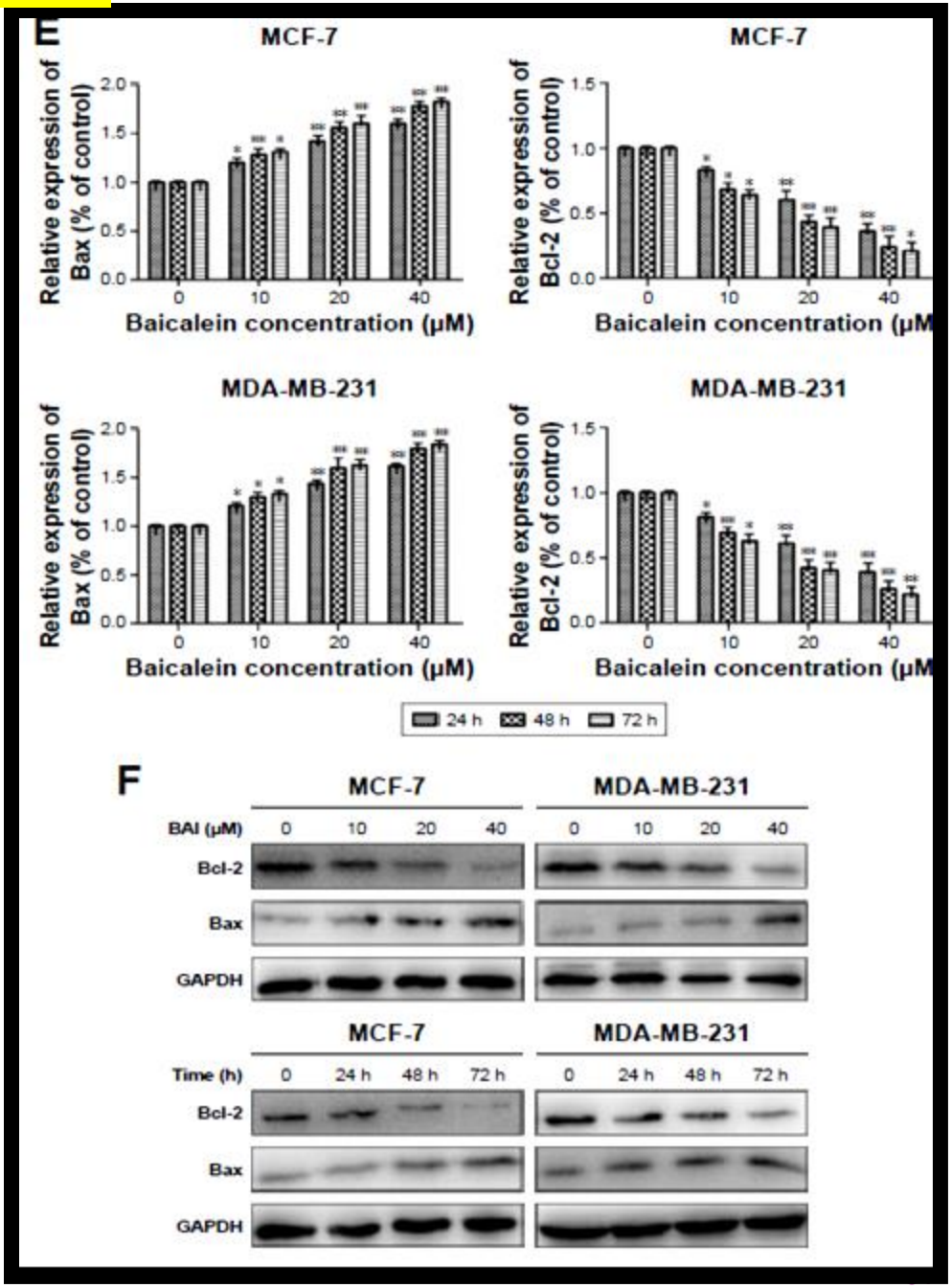
(Figure 2D).

- **qRT-PCR and Western blotting: the expression of apoptosis-related genes and proteins (Fig.2E & F):**
  - **↑ Bax & ↓ Bcl-2.**

Results


D
6.89%
13.08%
20.27%
26.89%
20.55%
27.73%
PI
Annexin V FITC
Control
10 μM
20 μM
40 μM
MCF-7
MDA-MB-231
Cell apoptotic rate (%)
Baicalein concentration (μM)
Early apoptotic rate
Late apoptotic rate
Total apoptotic rate

Results


E
MCF-7
Relative expression of Bax (% of control)
Baicalein concentration (μM)
MCF-7
Relative expression of Bcl-2 (% of control)
Baicalein concentration (μM
MDA-MB-231
Relative expression of Bax (% of control)
Baicalein concentration (μM)
MDA-MB-231
Relative expression of Bcl-2 (% of control)
Baicalein concentration (μM
24 h
48 h
72 h
F
MCF-7
MDA-MB-231
BAI (μM)
0
10
20
40
Bcl-2
Bax
GAPDH
MCF-7
MDA-MB-231
Time (h)
0
24 h
48 h
72 h
Bcl-2
Bax
GAPDH

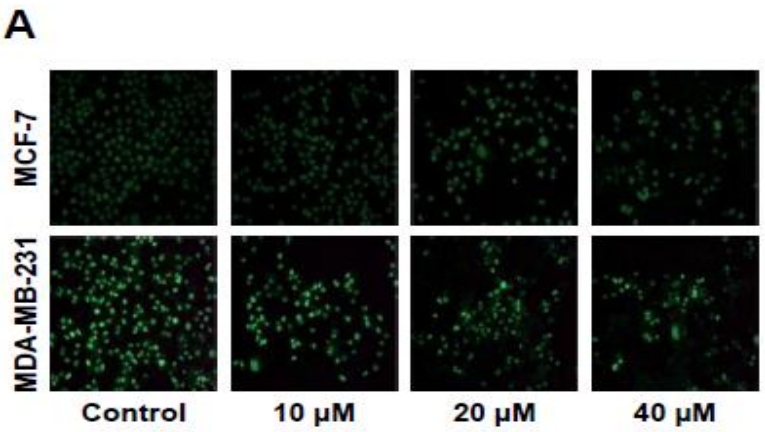
Results

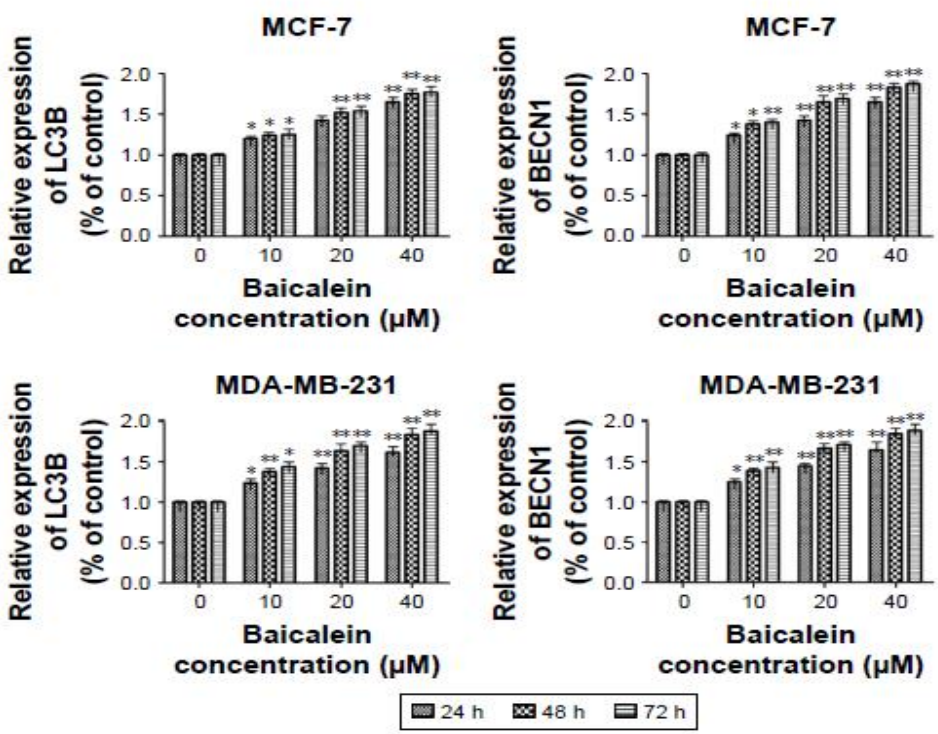
Baicalein induces autophagy in MCF-7 and MDA-MB-231 cells

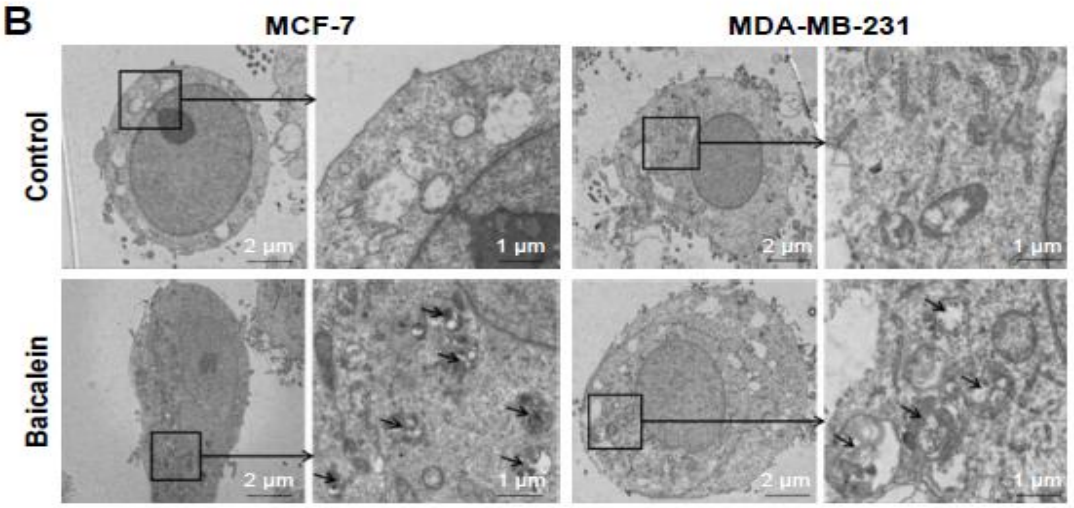
- **Baicalein induced the formation of autophagic vacuoles in MCF-7 and MDA-MB-231 cells in a dose-dependent manner (Fig.3A).**
- **Changes in morphological and ultrastructural features of cells were visualized. (Cells were treated with 40 μM baicalein for 48 hours) (Figure 3B).**

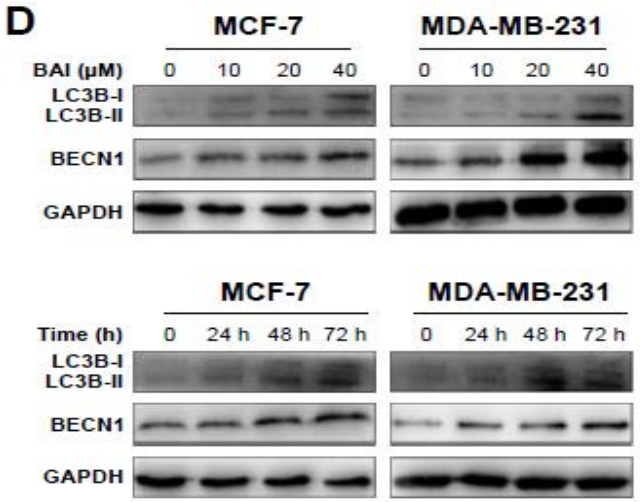
- **To further confirm the autophagy induced by baicalein, the expression of autophagy-related genes and proteins (LC3B and BECN1) was detected by qRT-PCR and Western blotting (Fig.3C and D).**

Results


A
MCF-7
MDA-MB-231
Control
10 μM
20 μM
40 μM


C
MCF-7
MDA-MB-231
Relative expression of LC3B (% of control)
Relative expression of BECN1 (% of control)
Baicalein concentration (μM)
0
10
20
40
24 h
48 h
72 h


B
MCF-7
MDA-MB-231
Control
Baicalein
2 μm
1 μm


D
MCF-7
MDA-MB-231
BAI (μM)
0
10
20
40
LC3B-I
LC3B-II
BECN1
GAPDH
Time (h)
0
24 h
48 h
72 h

Results

Baicalein regulates the apoptosis and autophagy of breast cancer cells via the PI3K/AKT/AKT signaling pathway

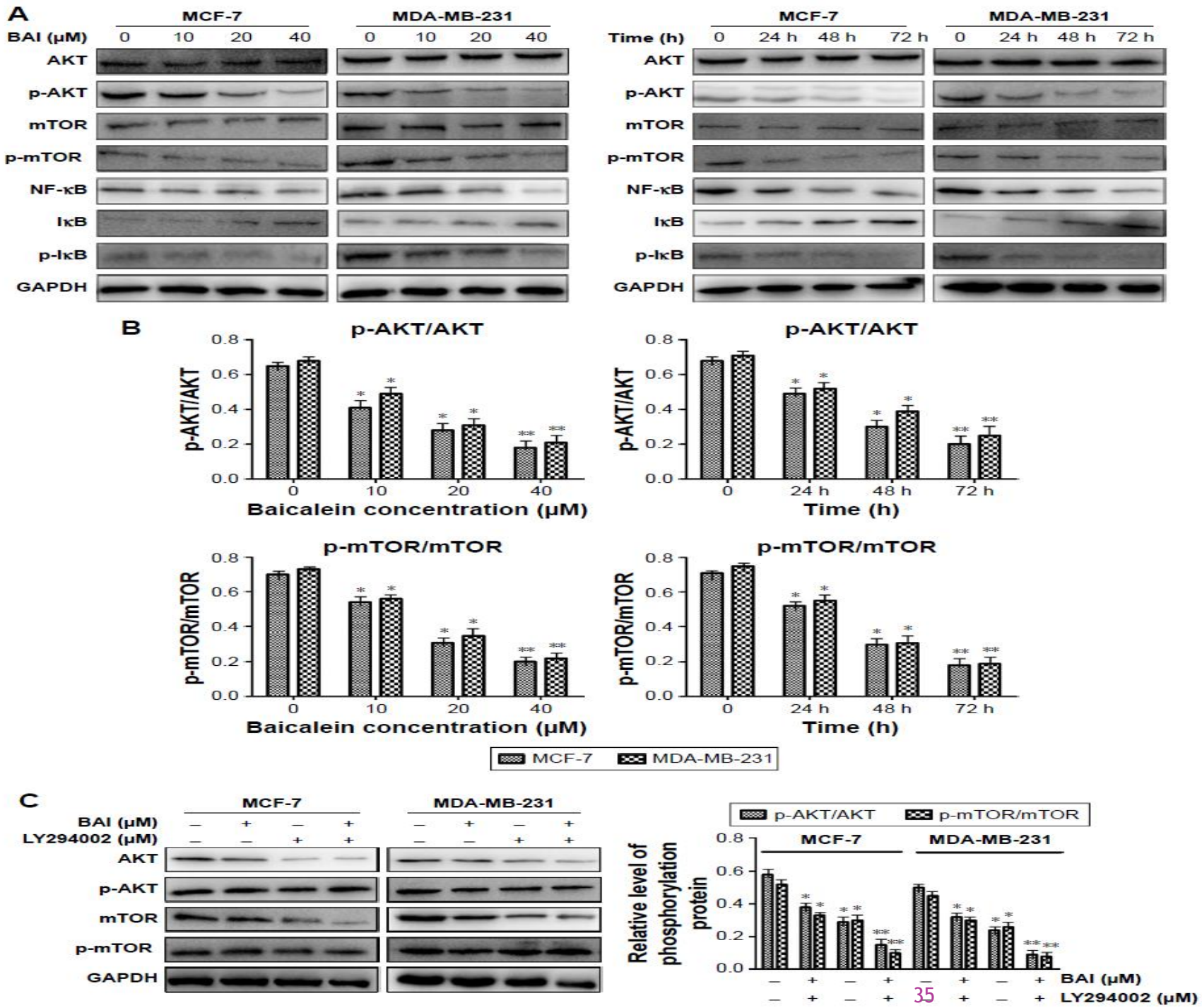
- **To elucidate the molecular mechanism of baicalein-induced apoptosis & autophagy, levels of proteins associated with the PI3K/AKT signaling pathway were examined using Western blotting:**

  p-AKT, p-mTOR, NF-κB, & p-IκB protein expression was ⇩

  p-AKT/AKT & p-mTOR/mTOR ratios ⇩ (Fig.4B).

- **To further confirm of the baicalein effects on PI3K/AKT signaling pathway: Breast cancer cells were treated with LY294002 (a specific PI3K inhibitor)➔ p-AKT & p-mTOR reduction (Fig.4C).**

- **These findings support the hypothesis that the induction of apoptosis and autophagy in cells by baicalein is mediated by the suppression of the PI3K/AKT signaling pathway.**


A
MCF-7
MDA-MB-231
BAI (μM)
0
10
20
40
Time (h)
24 h
48 h
72 h
AKT
p-AKT
mTOR
p-mTOR
NF-κB
IκB
p-IκB
GAPDH
B
p-AKT/AKT
p-mTOR/mTOR
Baicalein concentration (μM)
Time (h)
MCF-7
MDA-MB-231
C
LY294002 (μM)
p-AKT/AKT
p-mTOR/mTOR
Relative level of phosphorylation protein

Results

In vivo effects of baicalein on breast cancer xenograft model

- **In vitro: baicalein can inhibit proliferation and induce apoptosis and autophagy.**

- **In vivo: anticancer potential of baicalein were examined using breast xenografts models (BALB/c-nude).**

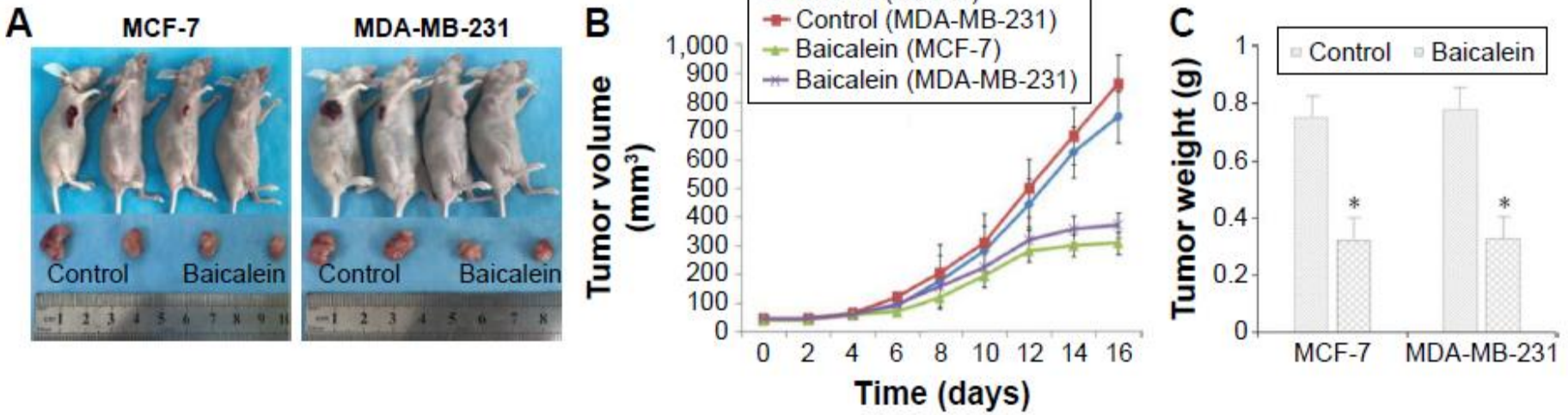
- **Treatment with baicalein➔ tumor tissues were collected and analyzed ➔**

  ⬇ growth, volume, and weight of tumors in the baicalein-treated group (Fig.5A–C)

- **Immunohistochemistry staining: baicalein: ⬇ p-AKT expression &**

  ⬆ expression of Bax & LC3 at the Pr level (Fig.5D)➔baicalein can induce apoptosis & autophagy via modulation of the PI3K/AKT pathway.

Results


A
MCF-7
MDA-MB-231
Control
Baicalein
Control
Baicalein
B
Control (MCF-7)
Control (MDA-MB-231)
Baicalein (MCF-7)
Baicalein (MDA-MB-231)
Tumor volume (mm³)
1,000
900
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16
Time (days)
C
Control
Baicalein
Tumor weight (g)
1
0.8
0.6
0.4
0.2
0
*
*
MCF-7
MDA-MB-231
D
MCF-7
MDA-MB-231
p-AKT
Bax
LC3
p-AKT
Bax
LC3
Control
Baicalein

Discussion

- **Previous in vivo & in vitro studies: baicalein induces apoptosis in lung cancer, cervical cancer, esophageal carcinoma and Burkitt lymphoma cells.**
- **There was a lack of information about the anticancer activity of baicalein in breast cancer, & on the signaling pathways related to apoptosis & autophagy.**

- **In present study established that baicalein:**
- **Inhibited cell proliferation in a time- and dose-dependent manner in MCF-7 & MDA-MB-231 cells by MTT and clone formation assays.**

- **Induced apoptosis in 2 breast cancer cell lines via Hoechst staining, ΔΨm, and annexin V-FITC assay.**

- **Induced apoptosis by increasing the Bax/BCL-2 ratio, as measured by qRT-PCR and Western blotting.**

Discussion

- **baicalein-induced autophagy assessment:**

- **The expression of BECN1 and LC3 was increased as shown by qRT-PCR and Western blotting.**

  (BECN1 and LC3, a central protein and the initiator of autophagy, are regarded as autophagy-related proteins (autophagosome formation & maturation)).

- **Autophagic cells were analyzes through acidic vesicular organelle staining assay, & autophagosomes detection under TEM.**

Discussion

- **The PI3K/AKT signaling pathway plays:**

- **A crucial role in regulating normal cell proliferation, differentiation, and apoptosis.**
- **Modulating the development & progression of cancers**
- **This signaling has been activated AKT, NF-κB, and mTOR(which are the downstream components of the PI3K/AKT pathway) ➔ when continuously activated ➔ maintenance of malignancies.**

- **Western blot were applied to evaluate the expression level of this proteins (AKT, p-AKT, mTOR, p-mTOR, NF-κB, IκB, and p-IκB) in the PI3K/AKT pathway.**

Discussion

- **Results: baicalein reduced the expression of p-AKT, p-mTOR, NF-κB.**
- **These findings further supported the hypothesis that the induction of apoptosis and autophagy in cells by baicalein is mediated by the suppression of the PI3K/AKT pathway.**
- **In addition, to acquire more reliable evidence to support and verify our in vitro experimental findings, we used the xenograft nude mouse model.**

Conclusion

Conclusion

- **baicalein had the potential to:**
- **suppress cell proliferation, induce apoptosis and autophagy in MCF-7 and MDA-MB-231 breast cancer cells via inhibiting the PI3K/AKT pathway both in vitro & in vivo.**
- **These results suggest that baicalein may have therapeutic potential for breast cancer treatment.**
- **The anti-tumor function of baicalein has not been investigated in clinical trials, further study of the mechanisms that underpin baicalein's anti-tumor activity may provide possible clinical applications in the treatment of breast cancer.**


thank
you